Effects of Hydroxyapatite Nanoparticles on Devitrification Crystallization in Cryoprotectant Solutions[1]

Lv Fukou，Liu Baolin，Li Weijie，Ganesh K. Jaganathan

Institute of Biothermal Science and Technology, University of Shanghai for Science and technology, shanghai 200093, China

Abstract: To study the effects of Hydroxyapatite (HA) nanoparticles on devitrification in cryoprotectant solutions, the crystallization of glycerol(60%,w/w) and PEG-600(50%,w/w) solutions with HA nanoparticles of different sizes (20nm、40nm、60nm）and different concentrations（0.1%、0.5%）during warming were investigated by using differential scanning calorimetry (DSC) combined with cryomicroscopy. Experimental results showed that the presence of nanoparticles does not change the glass transition temperatures and melting temperatures of quenched solutions, but affects the behavior of devitrification and recrystallization upon warming. The morphologies of glycerol and PEG-600 solutions are dendritic and spheric respectively, and the structures are not changed by adding nanoparticles. The ice fraction of glycerol solution containing 0.1% 60nm HA nanoparticles diminished significantly when comparing to the control solution. The ice fractions of PEG-600 solutions increased dramatically between -64℃ and -54℃. The findings have significant implications for biomaterial cryopreservation.

Key words Nanoparticles; Devitrification; Crystallization; DSC; Cryomicroscope

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0 Introduction

Vitrification is considered as the most feasible method for successful cryopreservation of cells, tissues and organs. This technique employs high concentration of low toxic cryoprotectants that suppresses the freezing point of water in the sample and freezing them at ultra-fast cooling rates to its glass transition temperature or below. As a result the water in the system is converted from liquid to glass state without crystallization. In regular conditions, two factors affect the probability of vitrification for a solution; the high concentration of cryoprotectant and the cooling rate. Achieving cooling rates of high orders is almost impractical in large sized samples due to the large thermal resistance. Amongst several ways to increase the freezing and warming rate during cryopreservation, use of nanoparticles holds promise. It has been reported that nanoparticles can enhance the heat transfer properties of solutions [1-2]. For example, Hao et al [3] showed that the thermal conductivity of Polyvinyl pyrrolidone (PVP) solutions with HA nanoparticles is larger than that of solution without nanoparticles even at cryogenic temperature.

In order to prevent ice crystal damage and increase the survival rate during cryopreservation of biological samples, it is necessary to have some knowledge concerning the crystallization during freezing and thawing. Many experimental studies have

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First author: Lv fukou (1979—), male, research assistant, Master, Research fields: cryopreservation of biomaterials.
Corresponding author: Liu baolin, Professor, blliuk@163.com

examined the behaviors of crystallization in aqueous solutions by using X-ray diffraction, cryomicroscopy and DSC or combination of these techniques. Using X-ray diffraction, Vigier [4] explored the mechanisms of the devitrification in water-glycerol mixture. As early as 1971, Diller [5] developed a cryomicroscopy to investigate the growth of ice-crystal during cryopreservation of biomaterial by tracking the ice front. Hey and MacFarlane [6-8] studied the isothermal and non-isothermal crystallization of water in glycerol and dimethyl sulfoxide (Me2SO) solutions using a combined DSC-video microscope technique. Bronshteyn and Steponkus [9] investigated the nucleation temperature and ice crystal growths in concentrated solutions of ethylene glycol (EG). Takehiko Gonda [10] studied the inhibitory effect of saccharides on ice crystals. Patrick Mehl [11,12] investigated the warming process in 1,2-propanediol aqueous solutions and the devitrification process of 1,3-butanediol aqueous solutions in isothermal and continuous heating rate (CHR) experiments by using DSC and cryomicroscope.

Most studies of cryopreservation have focused on the cooling process and its consequences. Publications often include some discussion of the warming effect, but there have been few experimental attempts to study the warming. Recent work suggests that the warming process is more important than cooling in cryopreserving biological function [13-14]. The purpose of this paper is to study the devitrification behaviors of two commonly used cryoprotectants namely glycerol (60% w/w) and poly(ethylene glycol) PEG-600 (50% w/w) solutions with and without Hydroxyapatite (HA) nanoparticles, and to investigate the effect of nanoparticles on devitrification crystallization during warming.

1 Materials and methods

Glycerol and polyethylene glycol (PEG-600) were purchased from Sinopharm Chemical Reagent Co., Ltd, and hydroxyapatite nanoparticles from Nanjing Emperor Nano Material Co., Ltd. The purity of glycerol and hydroxyapatite nanoparticles was 99.8% and 99.9% (w/w) respectively. The average molecular weight of PEG-600 was 570~630g/mol. HA nanoparticles of three sizes (20nm、40nm、60nm) were suspended at 0.1% or 0.5% (w/w) in 60% (w/w) glycerol and 50% (w/w) PEG-600 solutions prepared with purified deionized water. In order to get stable and uniform dispersions of nanoparticles, solutions were prepared with an ultrasonic vibrator. Nanoparticles were first added to the glycerol or PEG-600 solutions. The suspensions were placed in a water bath at 20±2˚C, and constantly agitated for 3h by a JY92-2D ultrasonic vibrator at 200watts. This procedure resulted in a uniform distribution of nanoparticles in solutions.

To investigate the cooling and warming of cryoprotectants, 10-15μl of solution was hermetically sealed in aluminum DSC pan and loaded into a Pyris Diamond DSC (Perkin-Elmer, Waltham, MA, USA) supplied with nitrogen gas at 20ml/min. DSC had been calibrated using the crystal–crystal transition of cyclopentane at -135.06°C and the melting point of distilled water at 0°C before the experiments. Weight of the solution in sample pan was measured (to 1.0μg) with a microbalance (Beijing Sartorius Instrument Co., Beijing, China). Each DSC run consisted of rapid cooling to -160℃ from room temperature at 150℃ /min and held for 2 minutes to thermally equilibrate, and then warming to 20℃ at 10℃/min. The glass transition temperature (T_g) was defined as the extrapolated onset temperature of the glass transition step, the devitrification temperature (T_d) and the melting temperature (T_m) were defined as the value of exothermic peak and endothermic peak respectively. The experiment was repeated three times and results were averaged.

We used the cryomicroscopy system, which is made up of BCS196 Biological Cryo-stage working with a Linksys 32 system (Linkam Scientific Instruments Ltd, UK). A film of sample solution (~1μl) was placed between two transparent glass cover slides of 8-mm diameter on a sample holder that was then inserted in the cold stage. A high resolution optical camera (Olympus Microscope BX50, Olympus Optical Co., Japan) was used to record the images for analysis, and the microphotographs can be taken together with a temperature logger. The cold stage program was the same as the DSC protocol. Since the direction of crystal growth was limited by coverslips, ice crystals were restricted mainly to two-dimensional growth rather than three-dimensional one. Assuming that the ice distribution was uniform in the thickness direction of the sample, the ice fraction in volume-per-volume (v/v) was determined as A_i/A in area-per-area, where A_i and A ($2.9mm^2$) represent the area of the ice crystal and that of entire field of observation respectively. The size of individual crystals was calculated as the radius of a circle having the equivalent projected area of the crystal as is employed by Tomoaki Hagiwara [15].

2 Results and discussion

2.1 Effect of nanoparticles on phase transition temperatures

The phase transition temperatures of glycerol and PEG-600 solutions without and with HA nanoparticles are summarized in Table1. The presence of HA nanoparticles had little effect on the glass transition temperatures and melting temperatures of glycerol and PEG-600 solutions. In glycerol samples with nanoparticles, the devitrification temperature is generally higher than that of solution without nanoparticles. The devitrification temperature of glycerol solution containing 0.1% 40nm HA is 4°C higher than that of solution without nanoparticles.

Table. 1 Phase transition temperatures for glycerol and PEG-600 solutions without and with HA nanoparticles determined from DSC.

Solutions	sizes and contents	T_g (°C)	T_d (°C)	T_m (°C)	T_m-T_d
Glycerol	No nanoparticle	-111.5±0.4	-53.6±0.5	-37.5±0.5	16.1
	20nm, 0.1%	-111.2±0.3	-51.9±0.5	-38.0±0.7	13.9
	40nm, 0.1%	-111.1±0.4	-50.0±0.4	-37.5±0.6	12.5
	60nm, 0.1%	-111.2±0.3	-51.2±0.4	-38.2±0.8	13.0
	20nm, 0.5%	-111.4±0.3	-51.7±0.3	-37. 5±0.4	14.2
	40nm, 0.5%	-111.3±0.3	-52.2±0.5	-37.6±0.6	14.6
	60nm, 0.5%	-111.1±0.4	-50.7±0.4	-38.3±0.5	12.4
PEG-600	No nanoparticle	-92.4±0.3	-52.4±0.3	-16.3±0.5	36.1
	20nm, 0.1%	-92.6±0.4	-51.6±0.4	-16.6±0.6	35.0
	40nm, 0.1%	-92.5±0.4	-52.3±0.5	-16.7±0.4	35.6
	60nm, 0.1%	-92.6±0.3	-51.3±0.6	-16.4±0.6	34.9
	20nm, 0.5%	-92.7±0.5	—	-16.9±0.8	—
	40nm, 0.5%	-92.4±0.3	—	-16.4±0.8	—
	60nm, 0.5%	-92.6±0.5	—	-16.8±0.5	—

Note. T_g, glass transition temperature; T_d, devitrification temperature; T_m, melting temperature.

"—" not denoted because of the double devitrification peaks.

The increment of devitrification temperature reflects the nanoparticles strengthen the glass-state network so as to inhibit devitrification.

In general, devitrification and recrystallization readily occur between T_d and T_m. $T_m - T_d$, the temperature window for ice formation, could be narrowed in glycerol solutions with the addition of nanoparticles, which means the stability of solution was improved by adding nanoparticles, such as the stability of glycerol solution containing 0.5% 60nm HA increased by 23% when compared to their counterpart that had no nanoparticles.

2.2 Effect of nanoparticles on ice morphology

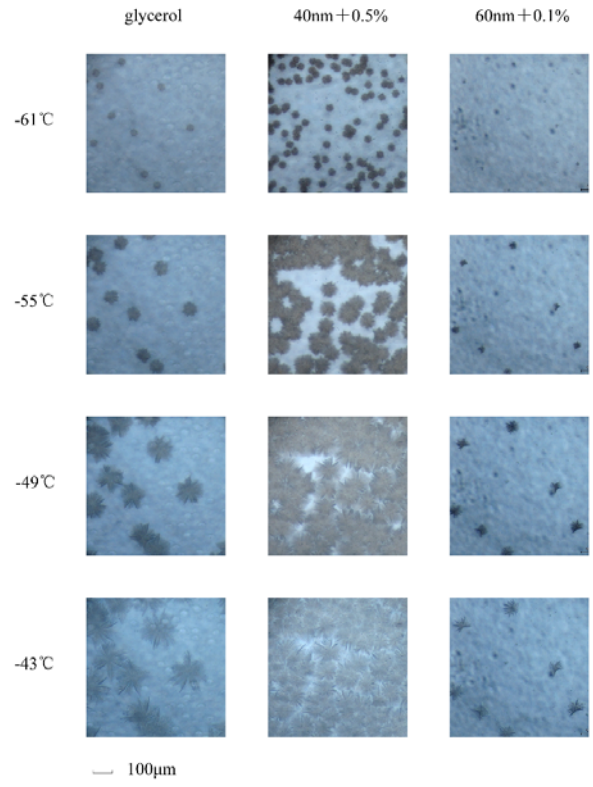

Fig. 1 Microphotographs of glycerol solutions (60% w/w) without nanoparticles, with 0.5% 40nm and 0.1% 60nm nanoparticles. Bar= 100μm

The typical microphotographs of ice crystal formed in glycerol and PEG-600 solutions during warming at 10°C /min are shown in Fig. 1 and Fig. 2 respectively. The morphologies of ice crystal are dendritic in glycerol solutions compared with the spheric in PEG-600 solutions, and are not transformed with the addition of nanoparticles. Glycerol is permeating cryoprotectant, while PEG-600 is non-permeating cryoprotectant, the morphology of ice crystal is mainly determined by the type of solution as is consistent with the result of Takehiko Gonda [10]. Ice morphology is crucial for preservation. Based on the ice formation theory, cells or interstitial structures are more vulnerable to mechanical damage caused by these "sharper" needle-shaped or dendritic crystals.

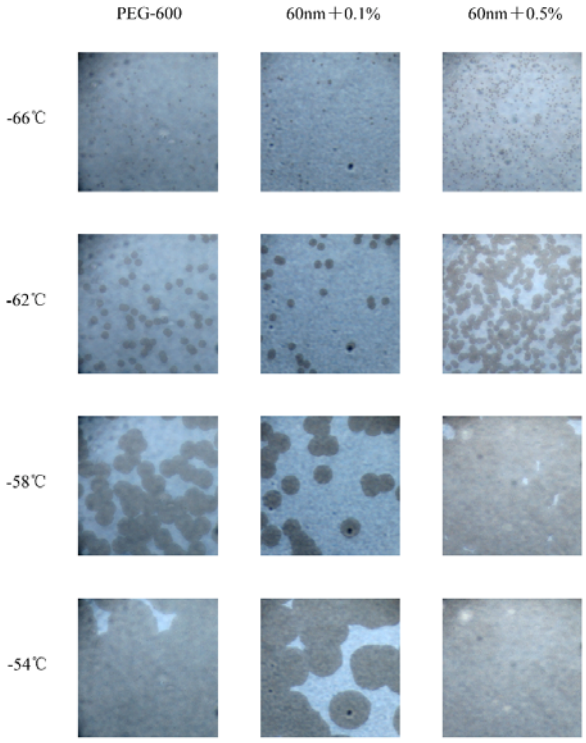

Fig. 2 Microphotographs of PEG-600 solutions (50% w/w) without nanoparticles, with 60nm HA whose concentrations are 0.1% and 0.5%.

2.3 Effect of nanoparticles on ice fraction

Fig. 3 and Fig. 4 show the change in ice fraction with temperature during warming at 10℃/min for glycerol and PEG-600 solutions. Ice fraction increased with warming for all the solutions, the trend is quicken first, and then slow down with the increasing

temperature until the end of recrystallization.

In the quenched glycerol solutions, 20nm and 60nm HA nanoparticles decreased, whereas 40nm HA nanoparticles increased the fraction of ice crystals at both 0.1% and 0.5% (w/w) in comparison to the control solution, and the ice fraction increased with higher nanoparticle concentration for the same size. The ice fraction of glycerol solution with 0.1% 60nm HA is 2/5 as that of solution without nanoparticles at -45℃. This is favorable for the cryopreservation because survival rate of biomaterial is decreased remarkably due to the osmosis of cell membrane, diminution of unfrozen water, toxicity of cryoprotectant solution, mechanical injury with the formation of ice crystal.

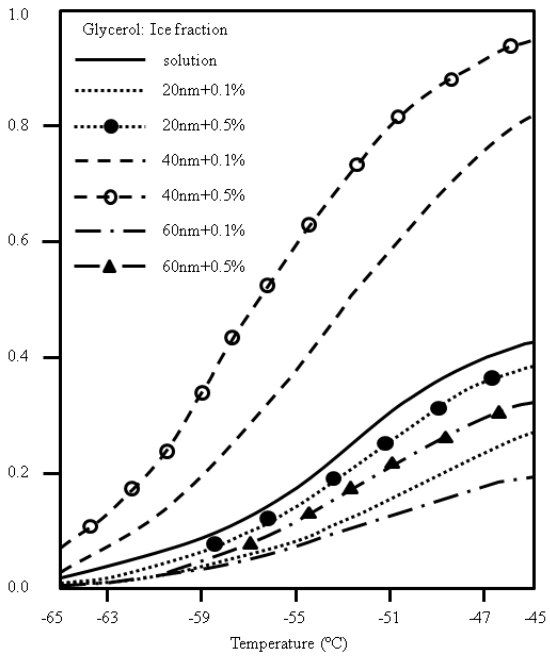

Fig. 3 Diagram of ice fraction with temperature during warming at 10℃/min for glycerol solutions with and without HA nanoparticles.

The ice fraction in PEG-600 solutions all increased to 1 by the end of recrystallization, that is to say, the ice crystal grow to the whole field of observation. The ice fraction of PEG-600 solutions increased remarkably between -64°C and -54°C, and the ice crystal fraction of PEG-600 solution without nanoparticles increased by 92% within the temperature range.

The liquid layering was generated on the surface of nanoparticles when we added nanoparticles into the cryoprotectant solution, as is explained by Hyun Uk Kang [16]. Not only the solute type, freezing and warming rate, mass and heat transfer but also the nanoparticles of different sizes and concentrations in the system, will alter the migration rate of water molecules, which result in the different behaviors of devitrification and recrystallization during warming, however, the exact mechanism seems to be unknown until now.

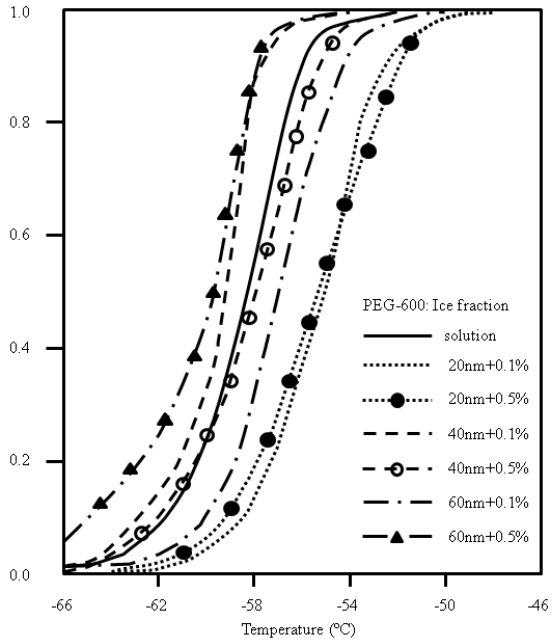

Fig. 4 Diagram of ice fraction with temperature during warming at 10℃/min for PEG-600 solutions with and without HA nanoparticles.

3 Conclusion

In conclusion, the presence of nanoparticles alters the behaviors of devitrification and recrystallization of quenched solutions upon warming. The explanation

and analysis for phase transition temperatures, ice morphology and ice fraction of glycerol and PEG-600 solutions during warming were presented. A better understanding of the molecular mechanisms will facilitate a systematic study of devitrification behaviors for various frozen samples. Since our systematic understanding of the interplay mechanism of nanoparticals and aqueous solution at subzero temperature is very limited until now, further studies are needed to solve this problem. Results of this study will be a basis regarding the application of nanoparticles in cryogenics and be useful for improving the survival rate of cryopreservation.

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